

# Sema4D, the Ligand for Plexin B1, Suppresses c-Met Activation and Migration and Promotes Melanocyte Survival and Growth

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Semaphorins are secreted and membrane-bound proteins involved in neural pathfinding, organogenesis, and tumor progression, through Plexin and neuropilin receptors. We recently reported that Plexin B1, the Semaphorin 4D (Sema4D) receptor, is a tumor-suppressor protein for melanoma, which functions, in part, through inhibition of the oncogenic c-Met tyrosine kinase receptor. In this report, we show that Sema4D is a protective paracrine factor for normal human melanocyte survival in response to UV irradiation, and that it stimulates proliferation and regulates the activity of the c-Met receptor. c-Met receptor signaling stimulates melanocyte migration, partly through downregulation of the cell adhesion molecule E-cadherin. Sema4D suppressed activation of c-Met in response to its ligand, hepatocyte growth factor (HGF), and partially blocked the suppressive effects of HGF on E-cadherin expression in melanocytes and HGF-dependent migration. These data demonstrate a role for Plexin B1 in maintenance of melanocyte survival and proliferation in the skin, and suggest that Sema4D and Plexin B1 act cooperatively with HGF and c-Met to regulate c-Met-dependent effects in human melanocytes. Because our data show that Plexin B1 is profoundly downregulated by UVB in melanocytes, loss of Plexin B1 may accentuate HGF-dependent effects on melanocytes, including melanocyte migration.

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## INTRODUCTION

Semaphorins are secreted or membrane bound proteins, and were originally described in the nervous system, but are also expressed in multiple organs, including the lung, kidney, bone, and lymph tissue (Takegahara *et al.*, 2005; Yazdani and Terman, 2006). Plexins, the transmembrane receptors for Semaphorins, are a family of highly conserved proteins that alone or in cooperation with neuropilins mediate the effects of Semaphorins (Tamagnone and Comoglio, 2000; Castellani and Rougon, 2002; Puschel, 2002; Fujisawa, 2004). The Plexin B1 receptor binds Semaphorin 4D (Sema4D), a class IV Semaphorin whose functions include neovascularization of tumors, axon guidance, and immune regulation (Elhabazi *et al.*, 2003; Ch'ng and Kumanogoh, 2010). Sema4D is cleaved by matrix metalloproteinases and is active in a membrane-bound and soluble form (Basile *et al.*, 2007; Zhu *et al.*, 2007). Plexin B1 has R-Ras and M-Ras GTPase activity (Oinuma *et al.*, 2004; Negishi *et al.*, 2005; Saito

*et al.*, 2009), and activates mitogen-activated protein kinase via Rho and integrin activation (Aurandt *et al.*, 2006; Oinuma *et al.*, 2006). Plexin B1 activation by Sema4D also participates in c-Met and ErbB receptor activation (Giordano *et al.*, 2002; Conrotto *et al.*, 2004; Wickramasinghe and Kong-Beltran, 2005; Swiercz *et al.*, 2008).

Melanocytes are critically important in the skin because they produce the pigment melanin, mitigating the effects of photoaging and photocarcinogenesis (Bhawan *et al.*, 1992; Tadokoro *et al.*, 2003; Wulf *et al.*, 2004). Many functions of melanocytes are regulated, in part, by growth factors produced by keratinocytes (Yaar *et al.*, 1991; Tada *et al.*, 1998; Imokawa, 2004; Cardinali *et al.*, 2005), or by the melanocytes themselves (Abdel-Malek *et al.*, 1999; Starner *et al.*, 2010). A potentially important role for Plexin B1 in melanocytes is suggested by recent reports showing that Plexin B1 is a tumor-suppressor protein for melanoma (Argast *et al.*, 2009; Stevens *et al.*, 2010). Plexin B1 expression is lost in melanoma *in vivo*, particularly in deeply invasive and metastatic tumors (Stevens *et al.*, 2010), and introduction of Plexin B1 into human melanoma cell lines abrogates metastasis in a mouse model (Argast *et al.*, 2009). Although the mechanism by which Plexin B1 suppresses melanoma progression is still being defined, we showed that Plexin B1 signaling blocks activation of the tyrosine kinase receptor c-Met by its ligand, hepatocyte growth factor (HGF) (Stevens *et al.*, 2010). c-Met controls multiple aspects of melanocyte function in response to HGF, which is

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Abbreviations: Erk, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; Sema4D, Semaphorin 4D; shRNA, short hairpin RNA

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upregulated by UV irradiation in keratinocytes and fibroblasts (Brenner *et al.*, 2005; Mildner *et al.*, 2007). c-Met signaling suppresses expression of E-cadherin in melanocytes, melanoma, and other cell types, and loss of E-cadherin contributes to melanocyte migration and progression of melanoma (Danen *et al.*, 1996; Davies *et al.*, 2001; Li *et al.*, 2001; Desiderio, 2007).

In this report, we examined the function of Plexin B1 in normal human melanocytes and the expression of Sema4D in the skin. Our data point to a role for Plexin B1 in melanocyte survival and proliferation, and suggest that loss of Plexin B1 may promote early stages of melanoma formation through enhanced c-Met activation, resulting in loss of E-cadherin expression and increased c-Met-dependent migration.

## RESULTS

### Sema4D and Plexin B1 are expressed in the skin *in vivo*

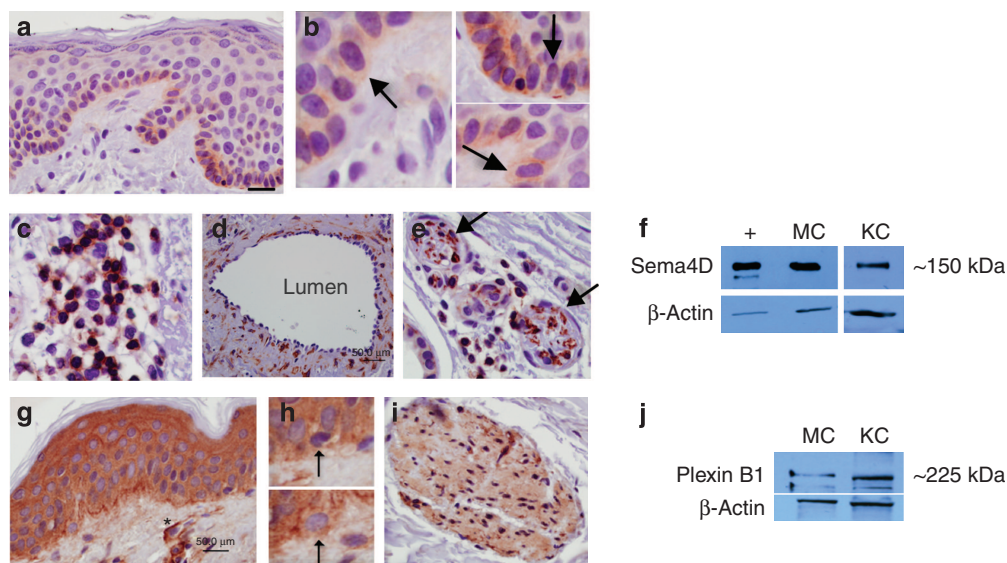
Immunocytochemical staining of skin shows strong expression of Sema4D in basal keratinocytes, closely apposed to melanocytes, which also expressed Sema4D, although weakly (Figure 1a and b); Sema4D was detected in dermal fibroblasts (not shown), lymphocytes (Figure 1c), smooth muscle of blood vessels (Figure 1d), and nerves (Figure 1e), but not in endothelial cells. Keratinocytes and melanocytes expressed Sema4D *in vitro* (Figure 1f). Immunocytochemical staining of skin showed strong diffuse expression of Plexin B1 in keratinocytes throughout all layers of the epidermis (Figure 1g). As previously reported, melanocytes express Plexin B1 *in vivo* (Figure 1h), as do endothelial cells (Stevens *et al.*,

2010). Smooth muscle of blood vessels and pilierector muscles also expressed Plexin B1 (Figure 1i). Western blotting of melanocyte and keratinocytes showed a band of the expected size ( $\sim 225$  kDa) in both cell types (Figure 1j) for Plexin B1, as well as a lower band ( $\sim 190$  kDa) likely representing the proteolytically processed receptor (Artigiani *et al.*, 2003). In some cases, a predominant band of  $\sim 100$  kDa was observed in melanocytes, as previously reported (Stevens *et al.*, 2010).

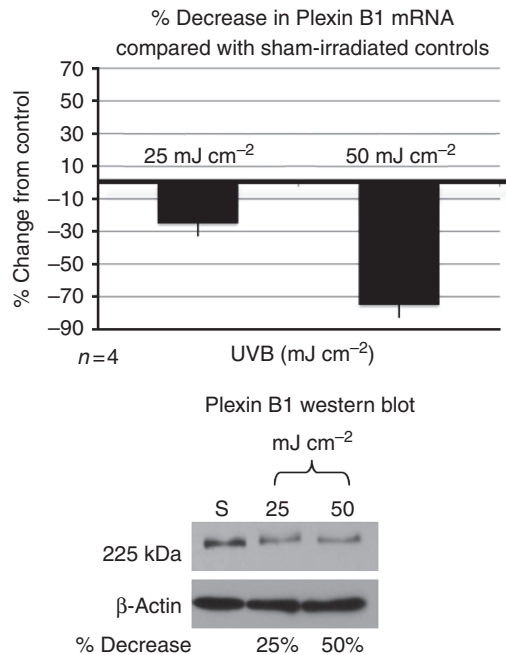
We next determined whether UVB regulates Plexin B1 in melanocytes (Figure 2). Message levels of Plexin B1 were lowered by 25 and 50%, 18 hours after 25 and 50 mJ cm<sup>-2</sup> UVB, respectively, compared with sham-irradiated controls. At 24 hours after irradiation, Plexin B1 at the protein level decreased 50% (25 mJ cm<sup>-2</sup>) and 75% (50 mJ cm<sup>-2</sup>) compared with sham-irradiated controls. UVB had no effect on Plexin B1 expression in keratinocytes or on Sema4D expression in melanocytes (data not shown).

### Sema4D is a survival factor for melanocytes

To determine whether Sema4D protects melanocytes from UVB-induced apoptosis, melanocytes were placed in basal medium overnight, and were pretreated with Sema4D (500 ng ml<sup>-1</sup>) for 8 hours, and then irradiated with UVB. The medium was replaced with fresh Sema4D; 18 hours later, floating cells were collected and combined with adherent cells, and levels of cleaved (activated) caspase-3 were determined by western blot analysis (Figure 3a). Sema4D abrogated UV-dependent caspase-3 activation at both doses of UVB by >30%. We knocked down Plexin B1 expression



**Figure 1. Semaphorin 4D (Sema4D) and Plexin B1 are expressed in the skin.** (a) Basal keratinocytes strongly express Sema4D (bar = 10  $\mu$ m). (b) Melanocytes, characterized by perinuclear halos, also express Sema4D (arrows, inset). (c) Lymphocytes, (d) smooth muscle, and (e) cutaneous nerves (arrows) express Sema4D. (f) Total cell lysates of melanocytes (MCs) and keratinocytes (KCs) were blotted for Sema4D (80  $\mu$ g loaded; 7.5% SDS-PAGE). A band of the expected size is detected in both cell types (representative of four experiments). Positive control (+) is a human squamous cell carcinoma cell line. The lower band ( $\sim 120$  kDa) likely represents the proteolytically cleaved portion of the protein. (g) Plexin B1 is expressed throughout all layers of the epidermis (bar = 50  $\mu$ m). Endothelial cells (asterisks) express Plexin B1, as do melanocytes (h: inset). (i) Smooth muscles of pili-erector apparatus also express Plexin B1. (j) Total cell lysates of MCs and KCs were resolved on 7.5% SDS-PAGE and blotted for Plexin B1 (40  $\mu$ g loaded). A band of the expected size is detected in both cell types (representative of four experiments).



**Figure 2. UVB downregulates Plexin B1 in melanocytes.** Melanocytes (MCs) were irradiated with UVB, or sham irradiated, and 18 hours later Plexin B1 was assessed by real-time PCR. Matched cultures were irradiated and Plexin B1 protein was measured 24 hours later by western blotting. The bar graph shows the average percent decrease in Plexin B1 mRNA compared with sham-irradiated cells,  $\pm$  SD (average of four cultures). The western blot shows lysates from a representative culture resolved on 7.5% SDS-PAGE and blotted for Plexin B1. The numbers beneath the blot indicate the percent decrease in Plexin B1 expression, normalized to  $\beta$ -actin.

in melanocytes and examined apoptosis by TUNEL assay in response to UVB. Plexin B1 knockdowns demonstrated significantly ( $P < 0.01$ ) more apoptosis in sham-irradiated cells compared with nontarget controls, which was further increased by irradiation (Figure 3b), in the absence of exogenous Sema4D. Growth factor-dependent activation of Akt suppresses apoptosis in melanocytes (Larribere *et al.*, 2004; Oka *et al.*, 2004; Kadekaro *et al.*, 2005). Sema4D transiently induced Akt activation in serum-starved nontarget controls, which peaked at 10 minutes. Knockdown of Plexin B1 completely blocked Sema4D-dependent Akt activation, indicating that Sema4D activates Akt through Plexin B1 (Figure 3c).

#### Plexin B1 signaling promotes melanocyte proliferation

Plexin B1 knockdowns grew very slowly, suggesting that Plexin B1 stimulates melanocyte proliferation. To test this, Plexin B1 knockdowns, or nontarget controls, were placed in basal medium for 8 hours, and then treated with Sema4D (100 ng ml<sup>-1</sup>) for 18 hours, and proliferation was assessed by CLICK-IT assay (Figure 4a). Proliferation was significantly lower in Plexin B1 knockdowns compared with cells expressing nontarget short hairpin RNA (shRNA), even in the absence of Sema4D. The addition of Sema4D modestly increased proliferation in nontarget controls, but this did not reach statistical significance, suggesting autocrine effects of

Sema4D. Analysis of extracellular signal-regulated kinase 1 and 2 (Erk1/Erk2) activation in response to Sema4D was performed in Plexin B1 knockdowns. Treatment of nontarget controls with Sema4D activated Erk1/Erk2, which peaked at 10 minutes (Figure 4b). Plexin B1 knockdowns showed lower basal levels of activated Erk1/Erk2, consistent with their low proliferative rate under resting conditions, and delayed and lower levels of Erk1/Erk2 activation in response to Sema4D compared with nontarget controls. Lack of complete blockade of Erk1/Erk2 activation in Plexin B1 knockdowns may be secondary to incomplete knockdown of the receptor (Supplementary Figure S1 online).

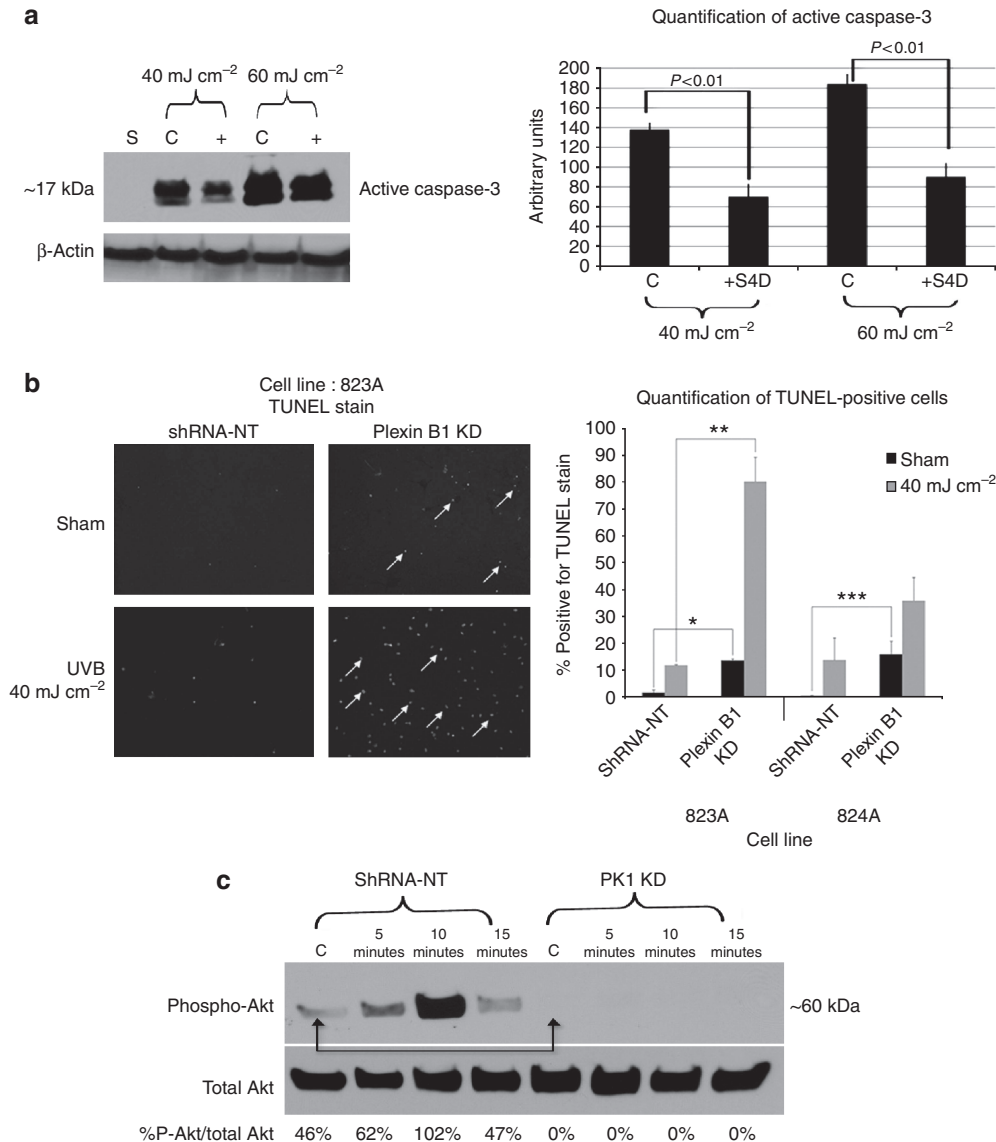
#### Sema4D blocks c-Met activation

To determine whether Sema4D blocks c-Met activation in melanocytes, cells were placed in basal medium overnight, pretreated with Sema4D (100 ng ml<sup>-1</sup>) for 30 minutes, and then treated with HGF (10 ng ml<sup>-1</sup> for 15 minutes) in the presence of Sema4D or control Sema4D (Figure 5a). Cells treated with HGF in the presence of control Sema4D stimulated c-Met phosphorylation 2.5-fold. In contrast, c-Met phosphorylation was only 1.8-fold in cells treated with Sema4D and HGF, a difference that was significant ( $P < 0.05$ ). Treatment of melanocytes with Sema4D alone had no effect on c-Met activity (Supplementary Figure S1 online), similar to what we observed in melanoma (Stevens *et al.*, 2010). Silencing of Plexin B1 blocked the effects of Sema4D on HGF-dependent c-Met activation, and resulted in higher basal levels of activated c-Met (Figure 5b).

HGF stimulates melanocyte migration, in part through downregulation of E-cadherin expression (Li *et al.*, 2001; Han *et al.*, 2005). We next determined whether Sema4D abrogates the effects of HGF on E-cadherin expression and migration. A dose response for HGF-dependent suppression of E-cadherin showed that doses as low as 5 ng ml<sup>-1</sup> completely suppressed E-cadherin expression in melanocytes (Supplementary Figure S2 online). We next treated melanocytes with HGF (10 ng ml<sup>-1</sup>) in the presence of Sema4D (100 ng ml<sup>-1</sup>) for 24 hours, and E-cadherin levels were analyzed (Figure 5c). Sema4D partially restored E-cadherin expression in melanocytes in response to HGF. We next determined whether Sema4D blocks HGF-dependent migration of melanocytes (Figure 5d). Melanocytes were grown to 80% confluence in six-well dishes, and migration was quantified by scratch assay at 24 hours. As expected, HGF (10 ng ml<sup>-1</sup>) stimulated melanocyte migration with an average fold increase of 4.36 ( $\pm 1.72$  SD) compared with vehicle-treated cells. In contrast, in cells treated with HGF and Sema4D (100 ng ml<sup>-1</sup>), migration was only 1.2-fold more ( $\pm 0.75$  SD) compared with vehicle-treated cells. The difference in fold change in migration between HGF/control and HGF/Sema4D-treated cells was significant ( $P = 0.04$ ,  $n = 3$ ). Sema4D alone (100 ng ml<sup>-1</sup>) had no effect on melanocyte migration.

#### DISCUSSION

We were motivated to conduct this study because Plexin B1 is a newly recognized tumor-suppressor protein



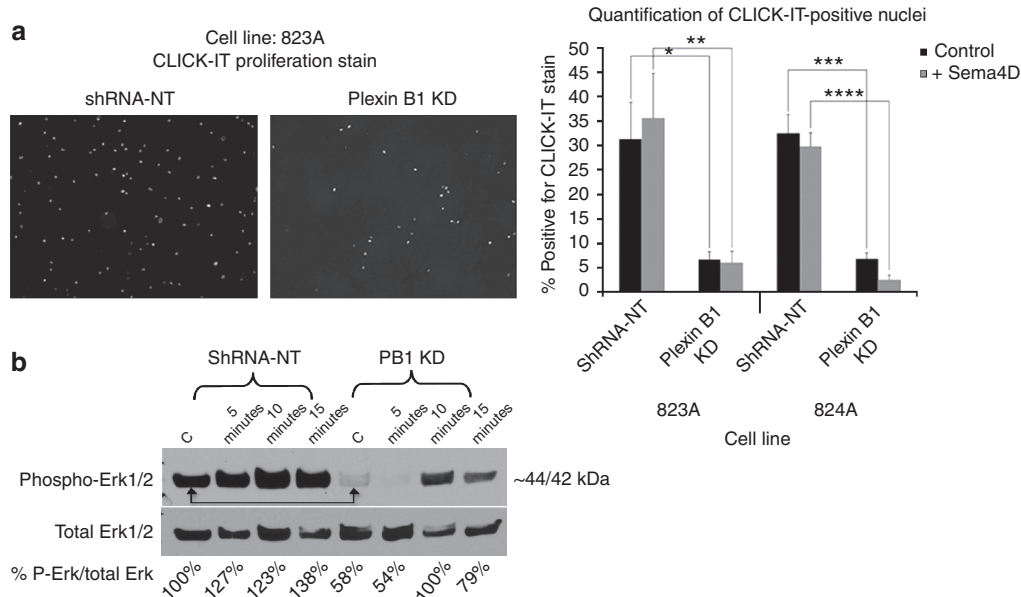
**Figure 3. Semaphorin 4D (Sema4D) protects melanocytes from apoptosis in response to UVB.** (a) Sema4D (+ S4D; 500 ng ml<sup>-1</sup>) abrogated UVB-dependent caspase-3 activation. Shown are total cell lysates of melanocytes resolved on 10% SDS-PAGE and blotted for active caspase-3. Results are representative of four separate experiments. Densitometry analysis of averaged ( $n = 4$  experiments) caspase-3 levels normalized to  $\beta$ -actin are shown. (b) Representative images of TUNEL staining of Plexin B1 knockdowns (KD) or controls (nontarget short hairpin RNA (shRNA-NT)) following UVR. Plexin B1 knockdowns show markedly higher levels of apoptosis, as shown by TUNEL-positive nuclei (arrows), in the absence of ligand. Following UVB, a striking increase in TUNEL-positive nuclei in Plexin B1 knockdowns was seen, compared with nontarget controls. Quantification of TUNEL-positive nuclei from melanocyte cultures silenced for Plexin B1 (823A and 824A) show highly significant differences compared with shRNA-NT controls. Each column represents the average of three separate experiments  $\pm$  SD. \* $P = 0.001$ ; \*\* $P = 0.0001$ ; \*\*\* $P = 0.0013$ . (c) Treatment of melanocytes with Sema4D (500 ng ml<sup>-1</sup>) stimulated Akt phosphorylation in control cells (shRNA-NT) but not in Plexin B1 knockdowns. Basal levels of phospho-Akt are lower in Plexin B1-silenced cells (arrows). Shown are total cellular lysates resolved on a 10% SDS-PAGE. Results are representative of three experiments.

for melanoma (Argast *et al.*, 2009; Stevens *et al.*, 2010). Understanding the function of this receptor in melanocytes could provide insight into how loss of the receptor promotes melanoma initiation or progression. In this report, we show that Plexin B1 expression is regulated by UVB in melanocytes, and that Plexin B1 signaling contributes to melanocyte growth and survival. Of particular interest was the observation that, similar to melanoma, Plexin B1 signaling suppresses c-Met activation and migration in response to HGF. Knock-

down of Plexin B1 increased basal levels of c-Met activation, suggesting that chronic loss of Plexin B1 may promote HGF-dependent migration and, potentially, early stages of melanoma progression through enhanced c-Met activity.

Sema4D is expressed by keratinocytes *in vivo*, suggesting that Sema4D is a paracrine factor for melanocytes. Because Sema4D stimulates angiogenesis, T-cell activation, and B-cell aggregation and viability, it may participate in new vessel formation or immune function in the skin, through effects on





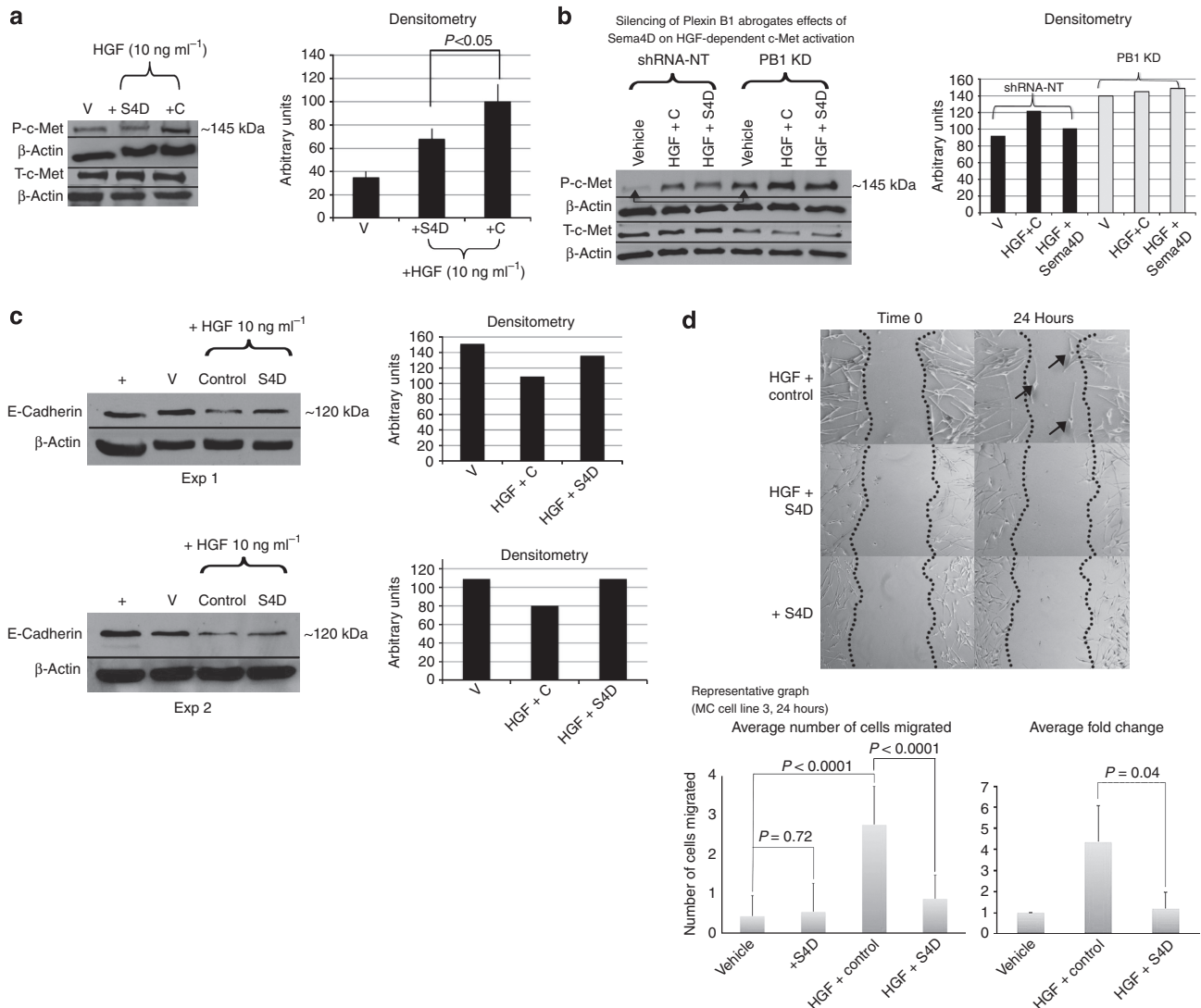
**Figure 4. Plexin B1 stimulates proliferation of melanocytes.** (a) Representative images of Plexin B1 knockdowns (KD) and control cells (nontarget short hairpin RNA (shRNA-NT)) from line 823A, stained to identify CLICK-IT uptake, and quantification of CLICK-IT-positive nuclei in both silenced cell lines (823A and 824A). Plexin B1 knockdowns showed a significant decrease in proliferation compared with controls. Each column represents the average of three separate experiments  $\pm$  SD. \* $P=0.005$ ; \*\* $P=0.006$ ; \*\*\* $P=0.0004$ ; \*\*\*\* $P=0.0001$ . (b) Treatment of melanocytes with Semaphorin 4D (Sema4D; 500 ng ml<sup>-1</sup>) activates extracellular signal-regulated kinase (Erk)1/Erk2, which peaks at 10 minutes. Knockdown of Plexin B1 delays and lowers Erk1/Erk2 activation in response to Sema4D. Basal levels of phospho-Erk1/2 are lower in Plexin B1-silenced cells (arrows). Shown are total cell lysates resolved on 10% SDS-PAGE; results are representative of three experiments. Controls consisted of cells treated with control Sema4D “(C)”.

vascular endothelium and lymphocytes. Sema4D was also expressed, although at lower levels, by melanocytes *in vivo* and *in vitro*. In our previous report (Stevens *et al.*, 2010), we did not identify Sema4D in melanocytes by western blotting. In this report, we were able to uncover expression by loading a larger amount of protein (80–100  $\mu$ g). Because of abundant expression of Sema4D by basal keratinocytes, it is likely that Sema4D is primarily a paracrine factor for melanocytes *in vivo*, although some autocrine effects may occur. Sema4D is released as an active product through the action of matrix metalloproteinases (Basile *et al.*, 2007); therefore, Sema4D from fibroblasts may function as a secreted paracrine factor for melanocytes, particularly following UV irradiation, which activates matrix metalloproteinases (Brenneisen *et al.*, 1996).

Sema4D protected melanocytes from UV-dependent apoptosis, as indicated by lower levels of cleaved caspase-3 in Sema4D-treated cells, similar to the effects of  $\alpha$ -melanocyte-stimulating hormone on UV-dependent apoptosis in melanocytes (Bohm *et al.*, 2005; Kadekaro *et al.*, 2005). Akt is a major effector of growth factor-dependent cell survival and was transiently activated by Sema4D in melanocytes. Whether transient activation of Akt by Sema4D has a role in protecting melanocytes from UVB-dependent apoptosis remains to be determined, but in human keratinocytes, short-term (1–2 hours) activation of Akt by IGF-1 delays the onset of UVB-dependent apoptosis at 48 hours (Decraene *et al.*, 2002). A similar pathway may be involved in the protective effects of Sema4D on apoptosis in response to UVB in melanocytes. In melanoma, Plexin B1 signaling also activates Akt and protects melanoma from apoptosis induced

by cis-platin (Stevens *et al.*, 2010). Therefore, Akt is a shared target of Plexin B1 in melanocytes and melanoma. Silencing of Plexin B1 profoundly inhibited proliferation in the absence of added Sema4D, suggesting potential autocrine effects, and treatment of melanocytes with Sema4D activated Erk1/Erk2 in a Plexin B1-dependent manner, consistent with a pro-proliferative effect of Plexin B1. In contrast, melanoma cell lines reconstituted with Plexin B1 proliferated slower than LacZ controls (Stevens *et al.*, 2010). Therefore, fundamental differences in Plexin B1 signaling on downstream pathways that control proliferation exist in melanocytes and melanoma.

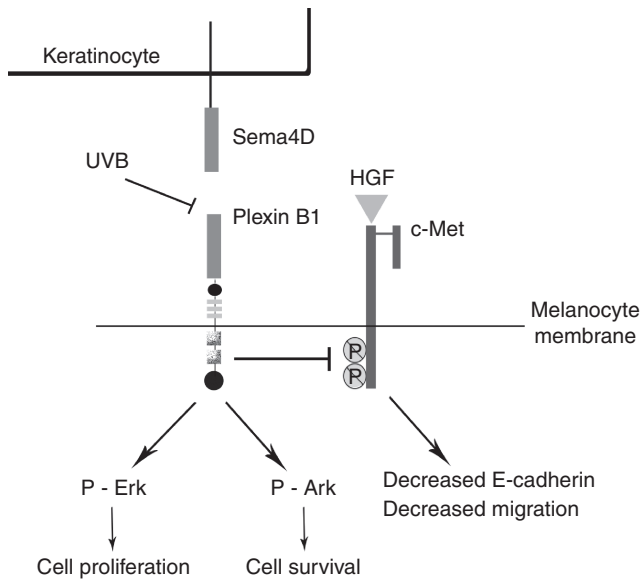
Our data show that Sema4D suppresses c-Met activation by HGF and partially blocks HGF-dependent E-cadherin downregulation and migration in melanocytes. Because Akt and Erk1/Erk2 are well-known targets of c-Met, we tested the ability of Sema4D to suppress HGF-dependent Akt and Erk1/Erk2 phosphorylation, but did not observe detectable changes in activation of these targets (Scott Laboratory, unpublished data). This may be because Plexin B1 and c-Met both activate Akt and Erk1/Erk2, masking the suppressive effects of Sema4D on HGF-dependent phosphorylation of these targets. Silencing of Plexin B1 blocked the inhibitory effect of Sema4D on HGF-dependent c-Met phosphorylation, and increased basal levels of activated c-Met, indicating that Plexin B1 is necessary for regulation of c-Met activity. The observation that basal levels of active c-Met are higher in Plexin B1 knockdowns suggests that autocrine production of Sema4D regulates the activity of c-Met in normal melanocytes. Alternatively, chronic loss of Plexin B1 may lead to compensatory changes resulting in activation of c-Met,



**Figure 5. Semaphorin 4D (Sema4D) inhibits hepatocyte growth factor (HGF)-dependent c-Met activation in melanocytes.** (a) Melanocytes treated with HGF (10 ng ml<sup>-1</sup>) for 15 minutes in the presence of Sema4D (S4D; 100 ng ml<sup>-1</sup>) show less phosphorylated c-Met compared with cells treated with HGF and control (C). Shown is a representative experiment in which total cell lysates were resolved on 10% SDS-PAGE. Densitometry levels of P-c-Met, normalized to β-actin, are shown. Each column represents the average of four separate experiments ± SD. (b) Control cells (nontarget short hairpin RNA (shRNA-NT)) or Plexin B1 knockdowns (PB1 KD) were treated as described in a. Shown is a representative experiment (n = 3) in which total cell lysates were resolved on 10% SDS-PAGE and blotted for P-c-Met. Densitometry analysis of P-c-Met, normalized to β-actin, is shown. Silencing of Plexin B1 blocked the suppressive effects of Sema4D on c-Met activation in response to HGF. In the absence of HGF ("Vehicle", arrow), silencing of Plexin B1 led to constitutive activation of c-Met. (c) Melanocytes treated with HGF (10 ng ml<sup>-1</sup>) for 24 hours show decreased E-cadherin expression compared with vehicle-treated controls, which is partially rescued by Sema4D (100 ng ml<sup>-1</sup>). Shown are two representative experiments of a total of three, and densitometry in which E-cadherin is normalized to β-actin. (d) Representative images from melanocytes from a scratch assay of cells treated with vehicle, HGF (10 ng ml<sup>-1</sup>) and control, or HGF (10 ng ml<sup>-1</sup>) and Sema4D (100 ng ml<sup>-1</sup>). Cells treated with Sema4D (S4D) alone are also shown (bottom). Melanocytes treated with HGF/control show more cells migrating into the scratch area (arrows), compared with cells treated with HGF/Sema4D. Sema4D alone had no effect on migration. Each column represents the averaged number of cells migrated into the scratch ± SD. The graph to the right shows the average fold change in migration from three experiments, with vehicle-treated cells set as "1" (± SD). Differences in fold change between HGF/C-treated cells and HGF/Sema4D-treated cells are significant (P = 0.04).

independent of ligand. The mechanism by which Plexin B1 inhibits c-Met activation in melanocytes remains to be defined. Several "co-receptors" for c-Met activation have been identified, including the adhesion molecules CD44v6 (Matzke *et al.*, 2007) and ICAM-1 (Olaku *et al.*, 2011), EGFR (Bergstrom *et al.*, 2000), and neuropilin-1 and neuropilin-2 (Sulpice *et al.*, 2008), which potentiate the activation of c-Met. To our knowledge, Plexin B1 is the first receptor

identified that stimulates and inhibits c-Met activation in a cell-type-specific manner. Because Plexin B1 shares significant homology with c-Met, it may compete with c-Met for binding to HGF, similar to neuropilin receptors (Sulpice *et al.*, 2008). Alternatively, Plexin B1 may form a complex with c-Met, preventing tyrosine phosphorylation of residues necessary for docking of adaptor proteins necessary for c-Met downstream effects, such as migration. The mechanism



**Figure 6. Proposed model of effects of Semaphorin 4D (Sema4D) on human melanocytes.** The Plexin B1 receptor on the melanocyte membrane binds Sema4D, which is produced by basal keratinocytes. Plexin B1 signaling activates Akt and extracellular signal-regulated kinase (Erk)1/Erk2, promoting melanocyte survival and proliferation, respectively. Exposure of melanocytes to UVB downregulates Plexin B1 expression, releasing inhibitory signaling on the c-Met receptor, which is activated by hepatocyte growth factor (HGF) produced by keratinocytes and fibroblasts. This results in enhanced c-Met-dependent loss of E-cadherin expression, promoting melanocyte migration and, potentially, transformation to melanoma.

by which Plexin B1 receptor signaling inhibits c-Met activation and cell migration will be the focus of a separate report.

In our proposed model (Figure 6), we predict that Plexin B1 signaling in melanocytes is activated through Sema4D produced by basal keratinocytes and melanocytes, promoting survival and proliferation. Plexin B1 maintains a brake on c-Met signaling, and maintains levels of E-cadherin expression. Following irradiation, HGF production by keratinocytes and fibroblasts is increased (Brenner *et al.*, 2005; Mildner *et al.*, 2007) and Plexin B1 expression in melanocytes is suppressed, resulting in enhanced c-Met activity and loss of E-cadherin expression. Because loss of E-cadherin is an early event in transformation of melanocytes to melanoma, Plexin B1 could function as a tumor-suppressor protein for early stages of melanoma by blocking the effects of c-Met signaling on E-cadherin expression and melanocyte migration.

## MATERIALS AND METHODS

### Reagents

PureCol was obtained from Inamed Biomaterials (Freemont, CA); the BCA Protein Assay kit was obtained from Pierce Chemical (Rockford, IL). Rabbit polyclonal antibodies to  $\beta$ -actin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA); rabbit polyclonal antibodies to Plexin B1 were purchased from ECM Biosciences (Versailles, KY). Mouse mAbs to Sema4D were purchased from BD Transduction Laboratories (Sparks, MD). Mouse mAbs to c-Met, rabbit mAbs to phospho-Met (Y-1234/Y-1235), rabbit mAbs to phospho-AKT and total AKT, rabbit mAbs to phospho-Erk1/Erk2,

mouse mAbs to total Erk1/Erk2, and rabbit mAbs to caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against E-cadherin were purchased from R&D systems (Minneapolis, MN). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies and hepatocyte growth factor were purchased from Sigma (St Louis, MO). Full-range rainbow molecular weight marker was purchased from Amersham Life Sciences (Arlington Heights, IL). Polybrene was purchased from Santa Cruz Biotechnologies.

### Cells and cell culture

Neonatal foreskins were obtained according to the University of Rochester Research Subjects Review Board guidelines and were the source of cultured human melanocytes and keratinocytes. Human melanocytes were cultured in Opti-MEM (Gibco-BRL, Grand Island, NY) containing 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA),  $10^{-4}$  M iso-butyl-methylxanthine, Anti-Anti (Gibco-BRL), 2.5 nM cholera toxin, 0.1 mM dibutyryl cyclic AMP, and 25 ng ml $^{-1}$  phorbol ester. Keratinocytes were cultured in Keratinocyte Serum-Free Media (Gibco-BRL). All supplements, except fetal bovine serum and Anti-Anti, were purchased from Sigma. With the exception of scratch assays and experiments in which Plexin B1-silenced cells were used, pooled cultures of melanocytes initiated from two to three foreskins were used at passages 4–8. Each experiment was repeated at least three times with separate pooled cultures of cells.

### Silencing of Plexin B1 in human melanocytes

Melanocytes from individual foreskin cultures (designated “823A,” “824A,” “1230B”) were plated in MGM at  $10^5$  cells in a six-well plate. Cells were infected at 2.5 multiplicity of infection with MISSION Sigma Lentivirus particles expressing shRNA targeting two loci of human Plexin B1 (Clones TRCN0000061535 and TRCN0000061536). Cells infected with nontarget shRNA in Lentivirus were used as controls. Silenced cells were selected with puromycin dihydrochloride (Sigma) at a concentration of 2.5  $\mu$ g ml $^{-1}$ . Silencing was confirmed by reverse transcription-PCR and western blotting (Supplementary Figure S3 online).

### UV irradiation

Cells were maintained in complete media and irradiation was carried out in sterile phosphate-buffered saline using a bank of six FS20 sun lamps (Westinghouse, Cranberry Township, PA) that have >75% emission in the UV irradiation range (280–320 nm), with a peak emission of 313 nm, and <25% UVA rays (>320 nm). Schott WGII filter (Elmsford, NY) was used to remove UVC rays. Irradiation times (in seconds) were calculated by multiplying the output of the FS20 lamps (Watts cm $^{-2}$ ) determined using a IL1700 meter (Newberry Port, MA), by the desired dose (mJ cm $^{-2}$ ). This number was divided by 10. Total irradiation times ranged from 120 seconds (40 mJ cm $^{-2}$ ) to 150 seconds (60 mJ cm $^{-2}$ ).

### Western blotting

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl) with protease inhibitors (Boehringer Mannheim GmbH, Mannheim, Germany) and protein was quantified using bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA). Protein was resolved on SDS-PAGE gels and blotted using standard procedures. Visualization

of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical).

### Immunohistochemical staining

Staining for Sema4D and Plexin B1 was carried out on sections of normal human adult skin from sun-exposed areas, which were obtained according to the University of Rochester Research Subjects Review Board guidelines. Staining for Plexin B1 was performed as previously described (Stevens *et al.*, 2010). For Sema4D, an identical protocol was followed, except that slides were incubated in a different antigen retrieval buffer (sodium citrate pH 6.0) and slides were incubated with anti-Sema4D mouse mAbs (1/100). Negative controls consisted of mouse IgG or rabbit IgG (Sigma) instead of the primary antibody.

### CLICK-IT and TUNEL assays

Staining was performed on melanocytes cultured on PurCol-coated coverslips. Analysis of proliferation was performed using the CLICK-IT assay (Invitrogen, Carlsbad, CA). TUNEL uptake was detected using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI). CLICK-IT- or TUNEL-positive nuclei were visualized using a filter with excitation wavelength of 495 nm. Positive nuclei were counted in a minimum of 200 cells, and percent positive nuclei determined by dividing by total nuclei, identified by 4,6-diamidino-2-phenylindole counterstaining, and viewed with a filter with excitation wavelength of 341 nm.

### Scratch assay

Melanocytes were plated in full media at a density of  $3 \times 10^5$  cells in a six-well dish and allowed to grow to near confluence (~80%). Three scratches were made on the bottom of each well using a sterile 200  $\mu$ l pipette tip. Wells were washed gently with phosphate-buffered saline and replaced with media containing mitomycin C to prevent proliferation ( $5 \mu\text{g ml}^{-1}$ ; Fisher Scientific, Pittsburg, PA), and either HGF ( $10 \text{ ng ml}^{-1}$ ) with Control or HGF ( $10 \text{ ng ml}^{-1}$ ) with Sema4D ( $100 \text{ ng ml}^{-1}$ ). Controls consisted of cells treated with vehicle alone and mitomycin C. Digital images from an inverted phase contrast microscope were taken 0 and 24 hours later. Quantification of migrated cells was performed by counting the number of cells (defined as cells with nuclei) that migrated from the edges of the scratches using the ImageJ software, NIH (Bethesda, MD). Three fields from each scratch (a total of nine fields per condition) were analyzed, and the numbers were averaged.

### Reverse transcription-PCR and comparative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, and reverse transcription and real-time PCR for Plexin B1 was performed as previously described (Stevens *et al.*, 2010).

### Recombinant Sema4D-Fc

Recombinant human Sema4D was expressed as an Fc-tagged protein in pEFBos vector (kindly provided by Hitoshi Kikutani, Osaka University, Osaka, Japan) in 293FT cells (Invitrogen). Sema4D was purified from culture supernatants as previously described (McClelland *et al.*, 2011). Protein purity, quantity, and identity were assessed by silver staining of gels and by western blotting (Supplementary Figure S4 online). Controls consisted of culture supernatants

of 293FT cells that were treated identically as culture supernatants of transfected cells ("control"). Specifically, culture supernatants of untransfected 293FT cells were collected, floating cells were removed by centrifugation, and supernatant was incubated with protein-A-conjugated sepharose beads. Following elution from the beads, the sample was assessed by silver staining of gels and by western blotting (Supplementary Figure S4 online).

### Statistical analysis

Differences between means were analyzed using two-tailed Student's *t*-test. A *P*-value of  $<0.05$  was considered significant.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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